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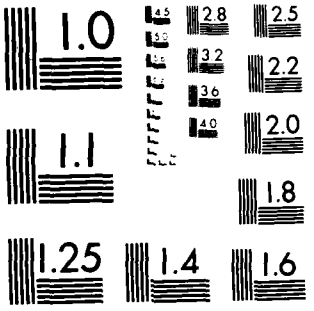

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ANNUAL REPORT

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Enzymatic Conversion of Red Cells for Transfusion

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Alpha-Galactosidase and Conversion of Group B Erythrocytes

Enzyme erythrocyte treatment conditions are being evaluated in order to determine the minimum amount of enzyme needed to convert the maximum number of cells within a time frame consonant with preserving cell viability and integrity. We have found that by increasing the cell concentration from 30% to at least 50% we can achieve conversion in 1-1.5 hours using 60-75 units of alpha-galactosidase per ml of packed erythrocytes. This is in contrast to our initial pre-clinical study the results of which were reported last year where 600 units of enzyme were used per ml of packed cells. In testing for enzyme reusability, we have found that the coffee bean alpha-galactosidase can be reused at least 11 times either in a continuous or discontinuous fashion, i.e. after freezing and thawing, without loss of activity and without any adverse effect upon the efficacy of the conversion process. This indicates that the reusability potential of coffee bean alpha-galactosidase is quite high. Contaminants from such an enzyme preparation can be removed by chromotography.

Since we plan to eventually convert whole units of group B erythrocytes for in vivo studies, we have begun to modify our current treatment procedures to accommodate processing of blood bank size batches of cells. Figure 1 outlines the treatment conditions we have used for small amounts of cells. For larger quantities, the effect of changes in the washing procedure (2), pH equilibration prior to incubation (3), incubation temperatures (4) and requilibration following conversion (6) are being evaluated. Thus far it appears that significant reduction in the number and time for both pre and post enzymatic conversion equilibrations can be made. Related to this is the effect of enzymatic conversion upon the viability and integrity of erythrocytes from blood stored 30-35 days. Our results show that incubation

under our treatment conditions for 1-1.5 hrs. does not significantly affect ATP levels of fresh or 30-day old stored cells. The level of 2, 3 DPG is totally depleted in 30-day old stored cells. However, it can be restored with rejuvinating solutions to levels greater than found in fresh cells. Treatment under our conditions does not change the level of 2,3 DPG in rejuvinated cells anymore than that for fresh cells (10-15%). Also, the increase in methemoglobin formation as a result of treatment for 1-1.5 hours is about the same for both fresh and 30-35 day old cells and remains metabolically insignificant. Stored cells (30-35 days) show less than 1% hemolysis as a result of treatment as do fresh cells. These results demonstrate that the conversion process does not impair the viability and integrity of stored cells anymore than that suffered as a result of their having aged in storage at 4°. Another related problem which we are studying in collaboration with Art Rowe and Leslie Lenny of the laboratory of Cryobiology at the New York Blood Center is the effect of enzymatic conversion upon fresh cells that are subsequently frozen either by the liquid nitrogen procedure or by mechanical means. Results thus far (see enclosed abstracts) indicate that enzymatic conversion and freezing by either method produced cells with ATP levels of 90% or greater and levels of 2,3 DPG at 70-80% of controls. Pretreatment with rejuvinating solutions results in 2,3 DPG levels at 100% or more of controls. It would appear then that enzymatic conversion of group B cells with or without rejuvination can be successfully combined with freezing using either of the two methods to yield O cells of transfusable quality.

Isolation and Characterization of Alpha-N-Acetylgalactosaminidase - (A-zymes)

We have searched for new sources of A-zyme in the microbial kingdom by screening microorganisms of known pedegree and bacteria selected at random from samples of soil. Those clones which can hydrolyze synthetic substrate i.e. p-nitrophenylglycoside of N-acetylgalactosamine and destroy blood group substance A activity are isolated and grown in sufficient quantities for us to isolate their A-zymes in partially purified form. These preparations are tested for activity under our pH conditions. We have been unable thus far to find a definite A-zyme in any microorganisms although in a few cases there was some destruction of A activity from the cell surface which could be attributable to contaminating endoglycosidic activity.

The first A-zyme we isolated from the animal kingdom came from the limpet Patella vulgata. Although it can remove A antigenicity from intact red cells, it functions too inefficiently under our conditions to be useful. We have now found an A-zyme in pigeon and chicken livers which is much more efficient in removing A antigenicity. The enzyme from chicken liver has been purified to the point where it is free of exoglycosidases (including neuraminidase) except for alpha-galactosidase which is present at 5-7% of the activity of the A-zyme at pH 5.7 and 32°. This partially purified A-zyme preparation can, as shown in Figure 2, convert group AB cells to HB without apparently affecting the level of B antigenic activity. Similarly P_1 antigenicity (also expressed by a terminally alpha-linked galactose residue) as well as Rh and M, N antigenicity are all unaffected by treatment with this A-zyme preparation. Preliminary studies also indicate, as shown in Table 1, that the time needed for conversion of group A cells to O is dependent upon the concentration of enzyme used for conversion. Isolation of larger quantities

and further purification of this alpha-N-acetylgalactosaminidase from chicken livers is currently being carried out. We then plan to perform in vitro metabolic and membrane studies with this A-zyme as described in the accompanying Renewal Proposal which would be analogous to those performed using the coffee bean B-zyme.

FIGURE 1

TREATMENT CONDITIONS

(1) TYPE B DONOR RED BLOOD CELLS COLLECTED IN CPDA-1

(2) PACKED CELLS WASHED 3 X WITH ISOTONIC SALINE.

(3) CELLS EQUILIBRATED 3 X AT 5' INTERVALS WITH PHOSPHATE-CITRATE-SODIUM CHLORIDE BUFFER pH 5.7.

(4) CELLS INCUBATED AT 26^{0} WITH GENTLE MIXING.

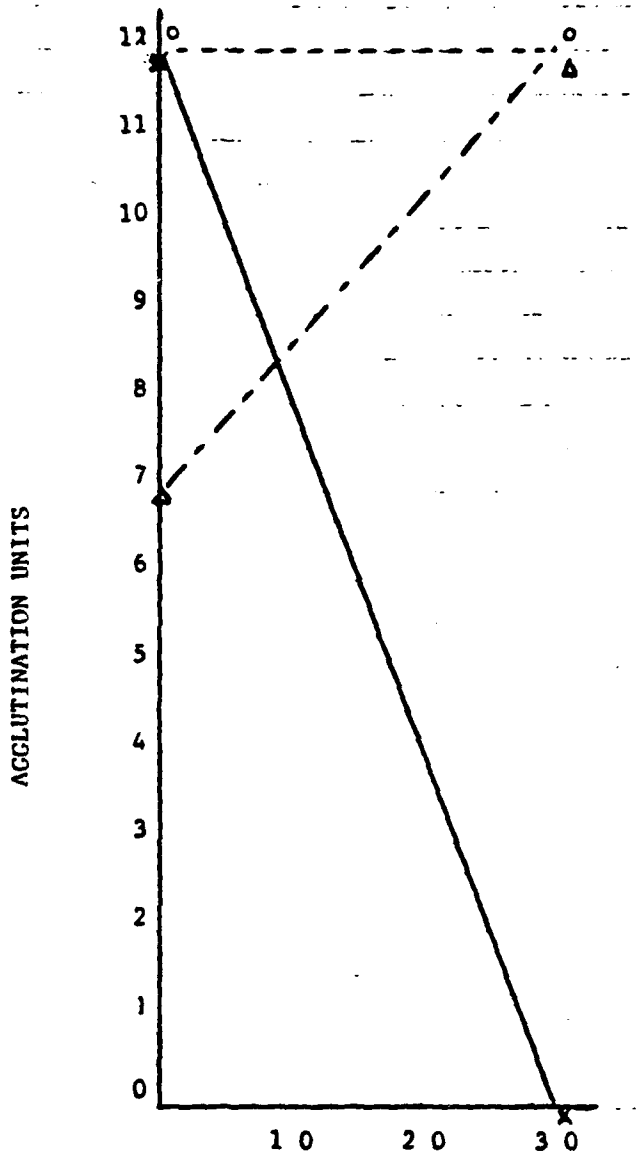
(5) ALIQUOTS REMOVED AT VARIOUS TIMES AND TESTED FOR CHANGES IN B AND H ACTIVITY.

(6) FOLLOWING INCUBATION CELLS ARE WASHED WITH PBS pH 7.4 AND ALLOWED TO COME TO pH EQUILIBRIUM (30').

Figure One is a Viewgraph and is complete
per Dr. Majde, ONR/Code 441

Figure 2

ENZYMATIC CONVERSION OF AB CELLS TO HB



0.1 ml AB cells at a final concentration of 64% incubated with 12 units of chicken liver alpha-N-acetylgalactosaminidase - pH 5.7, 32°

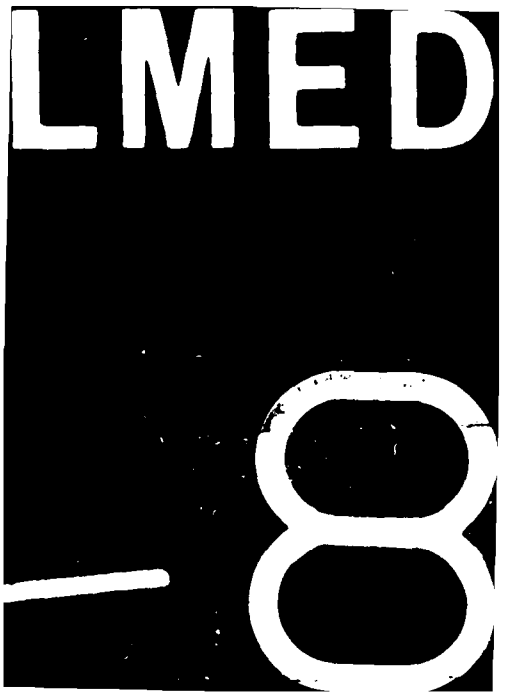
Table 1

RATE OF LOSS OF A ANTIGENIC ACTIVITY FROM

ENZYME-TREATED HUMAN RED CELLS

100 µl group A erythrocytes incubated with	30'	60'	90'	120'	180'
7 units Alpha-N-acetylgalactosaminidase	--	5	4	0	--
12 units " "	5	3	0	--	--
18 " " "	0	--	--	--	--

Cells at a concentration of 64% were incubated at pH 5.7, 32°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